J. Phycol. **35,** 1224–1232 (1999)

CA^{2+} BINDING PROTEIN CALRETICULIN IN *CHLAMYDOMONAS REINHARDTII* (CHLOROPHYTA): BIOCHEMICAL CHARACTERIZATION, DIFFERENTIAL EXPRESSION DURING SEXUAL REPRODUCTION, AND PHYLOGENETIC ANALYSIS[1]

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The occurrence of calreticulin, the main Ca^{2+} binding protein in the endoplasmic reticulum of eukaryotic cells, was investigated in the unicellular green alga *Chlamydomonas reinhardtii* Dangeard. The biochemical characterization of a diethylaminoethyl purified extract highlighted the presence, on SDS-PAGE, of a 55-kDa protein that stained blue with the Stains All dye, a diagnostic feature of acidic Ca^{2+} binding proteins. Immunoblot analyses revealed a strong cross-reaction of the *Chlamydomonas reinhardtii* protein with antibodies to plant calreticulins and the endoplasmic reticulum retention signal HDEL. Furthermore, the 55-kDa protein bound [$^{45}Ca^{2+}$] and had an acidic isoelectric point (pI = 4.9) but was neither glycosylated nor phosphorylated. N-terminal sequencing revealed strong amino acid sequence similarity to calreticulin from other sources. The presence of calreticulin in *Chlamydomonas reinhardtii* suggested that an endoplasmic reticulum Ca^{2+} buffering mechanism was present in this unicellular chlorophyte. The data suggest an early origin and high conservation of endoplasmic-reticulum-mediated Ca^{2+} functions in eukaryotes, whereby specific posttranslational modifications of the protein have been specifically acquired in different lineages of photosynthetic eukaryotes. Moreover, northern and western blot analysis experiments showed a regulation of calreticulin expression during *Chlamydomonas* sexual reproduction with a high abundance of calreticulin mRNA and protein in reproductive cells.

Key index words: **Ca^{2+} binding proteins; calreticulin; *Chlamydomonas reinhardtii*; endoplasmic reticulum; phylogeny; sexual reproduction**

Abbreviations: **AP, alkaline phosphatase; BiP, binding protein; CR, calreticulin; DEAE, diethylaminoethyl; DIG-11-dUTP, digoxigenin-11-2′-deoxy-uridine-5′-triphosphate; ER, endoplasmic reticulum; PVDF, polyvinylidene difluoride; TAP, tris-acetate-phosphate**

Intracellular Ca^{2+} stores are involved in the complex mechanism of Ca^{2+} homeostasis. Together with external Ca^{2+} sources, they control cytosolic Ca^{2+} concentrations. Intracellular Ca^{2+} stores transiently sequester Ca^{2+} and release it under cell stimulation. The endoplasmic reticulum (ER) is the major rapidly exchanging intracellular Ca^{2+} store in animal cells (Pozzan et al. 1994, Michalak 1996). In the lu-

[1] Received 17 July 1998. Accepted 30 July 1999.

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men of the ER, Ca^{2+} ions are buffered efficiently by Ca^{2+} binding proteins. Among them, calreticulin (CR) is one of the most predominant forms. CR is dynamically involved in Ca^{2+} storage as a high-capacity (~20 mol Ca^{2+}·mol^{-1} of protein) and low-affinity (K_d = 2 mM) Ca^{2+} binding protein (Baksh and Michalak 1996). Recent reports have attributed a multifunctional role to CR in the ER lumen, where it is not only involved in buffering free Ca^{2+} but also functions as a sensor of intraluminal free Ca^{2+} levels (Mery et al. 1996) and as a molecular chaperone assisting glycoprotein maturation (Coughlan et al. 1997, Tatu and Helenius 1997). Furthermore, CR has been implicated in the regulation of gene expression (Dedhar 1994, Masaeli 1996) and signal transduction (Coppolino et al. 1997, Zhu et al. 1997).

CR seems to be ubiquitous among eukaryotes: to date, yeast is the only organism in which the gene coding for CR has not been found (Krause and Michalak 1997). Recently, CR has been characterized in several higher plants, including dicotyledons and monocotyledons (Mariani and Navazio 1996), and recently, also in the photosynthetic protist *Euglena gracilis* (Navazio et al. 1998).

Comparisons of amino acid CR sequences from different primitive unicellular protists to higher plants and animals attest to a high level of structural conservation (Navazio et al. 1998). Among chlorophytes (most unicellular green algae and their multicellular descendants, Bhattacharya and Medlin 1998), the occurrence of CR and its role as an intracellular Ca^{2+} store in the ER have been investigated only in more advanced lineages of land plants. Functional and structural studies on CR from unicellular algae, among which the antecedents of land plants are sought, have been lacking. *Chlamydomonas reinhardtii* is a good model organism for many types of studies because of its simple organization and its physiological similarities to higher plants.

In this paper, we report the biochemical characterization of CR in *Chlamydomonas reinhardtii*. Evidence for the presence of CR in this alga indicates an origin of an ER-mediated Ca^{2+} buffering system at the unicellular level during chlorophyte evolution. Furthermore, recent findings revealing a regulation of CR expression during sexual reproduction in plants (Chen et al. 1994, Dresselhaus et al. 1996, Nelson et al. 1997, Williams et al. 1997) encouraged us to analyze the CR expression pattern during gametogenesis in *Chlamydomonas reinhardtii*.

MATERIAL AND METHODS

Cell cultures. Wild-type *Chlamydomonas reinhardtii* strain 137c (*mating type*$^+$, a kind gift of J. Girard, Institut de Biologie Physico-Chimique, Paris, France) were grown at 24° C in liquid tris-acetate-phosphate (TAP) medium (Harris 1989) with an initial density of $5 \cdot 10^6$ cells·mL^{-1}, under a 16:8 h LD photoregime. Gametogenesis was induced by transferring an aliquot of vegetative cultures in midlog phase to nitrogen-free TAP medium (Harris 1989) to a density of $5 \cdot 10^6$ cells·mL^{-1} and incubating with shaking at 24° C. Pregametes were obtained by dark incubation in nitrogen-free medium for 17 h. Mature gametes were generated with illumination of pregamete cultures for 8 h with shaking at 24° C. Light supplied by Osram L58W/21-840 Lumilux Plus (Milan, Italy) (350–700 nm) was used for illumination. Vegetative cells in the midlog phase, pregametes, and gametes were harvested by centrifugation (5 min, 2000 rpm) and immediately frozen in liquid nitrogen.

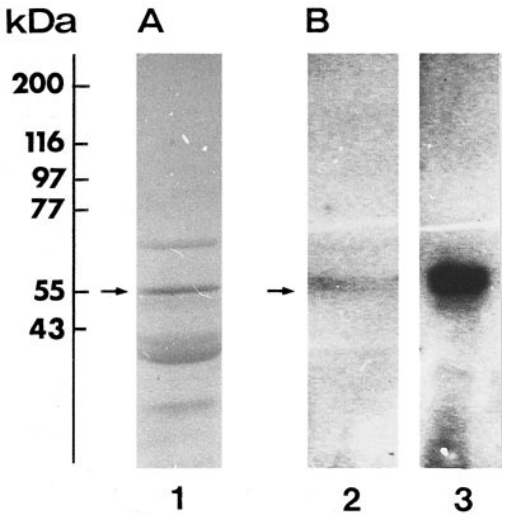

FIG. 1. Identification of *Chlamydomonas* Ca^{2+} binding proteins. SDS-PAGE of fraction eluted between 0.1 and 0.2 M NaCl from a DEAE-cellulose column chromatography were analyzed by Stains All staining of gel (A) and [$^{45}Ca^{2+}$] ligand overlay of blot (B, autoradiography exposed for 7 days). Lanes 1 and 2, *Chlamydomonas* protein fraction (15 μg); lane 3, spinach CR (30 μg). Arrows indicate blue-stained and Ca^{2+}-binding proteins.

Crude extracts. Crude extracts were obtained resuspending the harvested cells in 2 volumes·g^{-1} of cells of HEPES 10 mM/saccharose 0.32 M (Sigma, St. Louis, Missouri) buffer, containing the protease inhibitors phenylmethylsulphonylfluoride (0.5 mM) (Sigma) and benzamidine (0.5 mM) (Sigma) and breaking them with a French Press operating at a pressure of 1500 psi.

Protein isolation. For protein isolation, the pelleted cells were resuspended in 4 volumes·g^{-1} of cells of extraction buffer (1.0 mM EDTA, 0.1 M KH_2PO_4, 2.66 M $(NH_4)_2SO_4$, pH 7.1), containing the protease inhibitors phenylmethylsulphonylfluoride (0.5 mM) and benzamidine (0.5 mM) and broken with a French Press operating at a pressure of 1500 psi. The extraction of Ca^{2+} binding proteins was carried out at 4° C employing an ammonium sulfate precipitation step (Navazio et al. 1995) followed by diethylaminoethyl (DEAE)-cellulose (DE52, Whatman, Maidstone, U.K.) column chromatography, as reported by Milner et al. (1991). The column was eluted with a NaCl linear gradient (0.05–1.0 M) and 2.5 mL fractions were collected. Protein concentration was determined with the Protein Assay Reagent (Bio-Rad, München, Germany) using bovine serum albumin (BSA) as a standard (Bradford 1976).

Polyacrylamide gel electrophoresis and immunoblotting analyses. Protein profiles of fractions from DEAE-cellulose chromatography and from crude extracts were obtained by SDS-PAGE using a 7.5%–10% acrylamide linear gradient (Laemmli 1970). Gels were stained with Coomassie Brilliant Blue R-250 (Sigma), destained with 10% acetic acid/50% methanol, then restained with Stains All dye (Bio-Rad, Richmond, California), as described by Campbell et al. (1983). Following electrophoresis, proteins were transferred onto 0.2 μm nitrocellulose (Pharmacia, San Francisco, California) or polyvinylidene difluoride (PVDF) (Gelman Sciences, Milan, Italy) for chemiluminescent detection, according to Towbin et al. (1979). Membranes were probed with polyclonal antisera against CR from spinach (dilution 1:1500, Navazio et al.

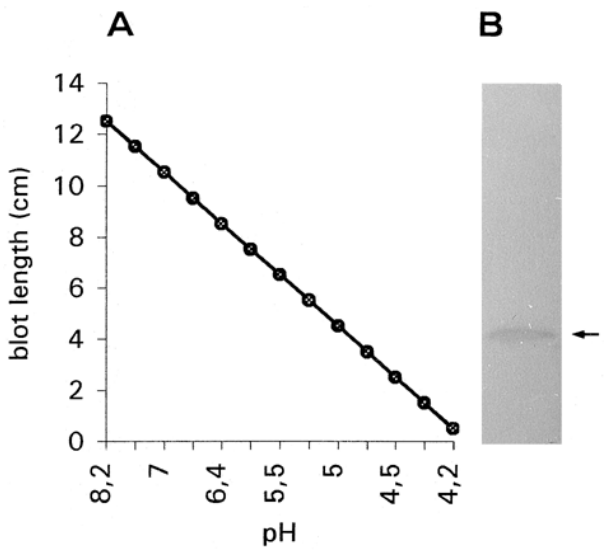

FIG. 2. Isoelectrofocusing of the 0.1–0.2 M NaCl DEAE-cellulose fraction. Proteins (15 μg) were transferred onto nitrocellulose membrane and probed with antiserum to spinach CR (B). Arrow indicates the *Chlamydomonas* Ca^{2+} binding protein. pI was calculated from the graph shown in (A).

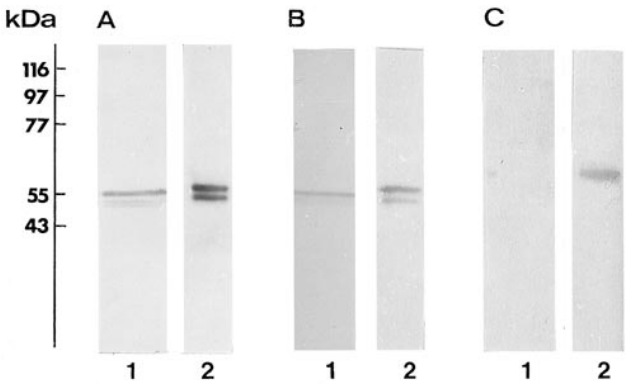

FIG. 3. Immunological analyses of the 0.1–0.2 M NaCl DEAE-cellulose fraction. Immunostaining of 0.1–0.2 M NaCl DEAE-cellulose fraction using antibodies to CR from spinach (A), maize (B), and rabbit (C). Lane 1A, 1B, and 1C, *Chlamydomonas* CR (15 μg); lanes 2A and 2B, spinach CR (2 μg); lane 2C, rabbit CR (2 μg).

1995), maize (1:5000, Napier et al. 1995), and rabbit (1:2000, from M. Michalak, Edmonton, Canada) and a polyclonal antiserum raised against tobacco binding protein (BiP) (1:2000, from A. Vitale, Milano, Italy). In addition, the monoclonal antibody 2E7 raised against the HDEL sequence was used (1:200, Napier et al. 1992). Immune complexes were detected by using an anti-rabbit IgG conjugated with alkaline phosphatase (Boehringer Mannheim, Monza, Italy). Chemiluminescent detection was carried out by incubating the PVDF membranes with CDP-star™ (Biolabs, Hitchin, UK) according to the manufacturers instructions. Then membranes were subjected to autoradiography (Fuji Films) for 5–15 min and the exposed films were quantified by densitometry using a Gel Doc 1000 and Molecular Analyst software (BioRad, Oakland, California). DEAE-cellulose fractions or pure CR isolated from spinach leaves and rabbit liver (Navazio et al. 1995) were employed as controls in both SDS-PAGE and immunoblot analyses.

Affinity detection and [$^{45}Ca^{2+}$] binding. Affinity detection of high-mannose glycans was carried out on blots by using horseradish peroxidase-conjugated Concanavalin A, as described by Faye and Chrispeels (1985). A [$^{45}Ca^{2+}$] ligand overlay assay was performed on the blot following the method described by Maruyama et al. (1984). The nitrocellulose membrane, after incubation in [$^{45}Ca^{2+}$], was subjected to autoradiography on Hyperfilm MP films (Amersham, Buckinghamshire, United Kingdom) for 7 days. CR isolated from spinach leaves (Navazio et al. 1995) was used as control.

Isoelectrofocusing. Isoelectrofocusing was carried out by using the procedure described by O'Farrell (1975) for the first dimension. The slab gel used contained 9 M urea and 2.0% ampholines (1.0% pH range 3.0–10.0, 0.5% pH range 4.0–6.5, 0.5% pH range 3.5–5.0). The gel was focused for 10 h at 350 V and, after equilibrating the gel in transfer buffer containing 0.01% SDS, proteins were transferred onto a nitrocellulose membrane that was probed with antiserum to spinach CR, as described above.

N-terminal sequencing. N-terminal amino acid sequence was determined by using gel bands, corresponding to the 55-kDa protein, excised from multiple lanes. These were loaded onto a funnel-shaped concentration gel and electroblotted onto a PVDF membrane according to Matsudaira (1987). Protein sequencing was carried out by using an Applied Biosystems model 476A sequencer (Foster City, CA).

Phosphorylation assay. CR samples were incubated in 50 mM Tris/HCl, pH 7.5, 0.12 M $MgCl_2$, 0.1 M NaCl, in the presence of 40 μM [γ-^{32}P]ATP and 10–50 mU of protein kinases CK2 and CK1 (Meggio et al. 1981) and tyrosine kinase Syk (a generous gift of A. M. Brunati, Padova, Italy) according to Cala and Jones (1991). Polylysine, when present, was 420 nM. Treatment with alkaline phosphatase (AP) was performed by incubation for 30 min at 37° C of CR samples with 2 μg of AP (Sigma). AP was inactivated by heat (5 min, 50° C), then samples were cooled on ice to stop the reaction and loaded on a 7.5%–10% SDS-PAGE gradient gel (Laemmli 1970). Gels were dried after staining with Coomassie Brilliant Blue (Sigma) and scanned on an Instant Imager Apparatus (Camberra-Packard, Downers Grove, Illinois).

RNA isolation and Northern hybridization. Total RNA from vegetative cells, pregametes, and gametes was isolated as described by Logemann et al. (1987). Thirty micrograms of total RNA were electrophoresed on a 1% agarose-formaldehyde gel and transferred onto a nylon Plus membrane (QIAGEN, Hilden-Germany) with SSC 20× solution. The membrane was prehybridized for 1 h at 42° C then hybridized overnight at 65° C with a digoxigenin-11-2′-deoxy-uridine-5′-triphosphate (DIG-11-dUTP)-labeled 800 bp fragment coding for CR (Zuppini et al. 1999) according to the "DIG High Prime DNA labeling and Detection starter kit" instructions (Boehringer). Quantification of hybridization signals was carried out by densitometry of the exposed film using Gel Doc 1000 equipment and the Molecular Analyst software (Bio-Rad).

Sequence data analysis. Sequences were obtained from GenBank and aligned with ClustalW (Thompson et al. 1994). Regions of the alignment that were highly variable or contained large numbers of gaps were excluded from analysis using criteria described (Hansen et al. 1999), leaving 350 amino acids sites that were used for phylogenetic inference (alignment available upon request). Numbers of substitutions per site were estimated using the Kimura distance option of PHYLIP (Felstein 1993) and were used to construct the neighbor-joining tree (Saitou and Nei 1987). Bootstrap proportions for branches were determined with the corresponding programs of PHYLIP; five hundred bootstrap samples were analyzed.

RESULTS

Biochemical characterization. Ca^{2+} binding proteins from *Chlamydomonas* vegetative cells (*mating type*$^+$) were extracted with the use of an ammonium sulfate precipitation procedure commonly employed in isolation of CR from animals (Milner et al. 1991) and plants (Navazio et al. 1995). Fractions obtained by DEAE-cellulose chromatography were analyzed by

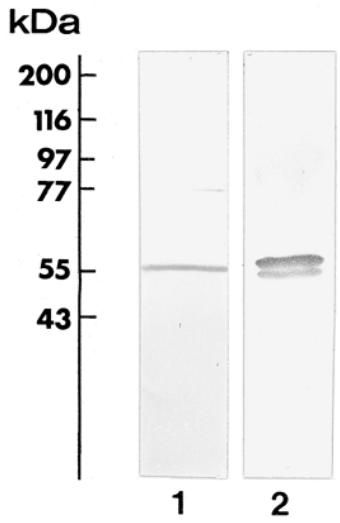

FIG. 4. Immunostaining of DEAE-cellulose fraction with an antibody to the HDEL retention sequence. Immunological cross-reactivity of the 0.1–0.2 M NaCl DEAE-cellulose fraction with a monoclonal antibody to the HDEL ER retention sequence. Lane 1, *Chlamydomonas* CR (15 μg); lane 2, spinach CR (40 μg).

SDS-PAGE. This procedure allowed us to detect a major 55-kDa band selectively staining blue with the Stains All dye in the fraction eluted between 0.1–0.2 M NaCl (Fig. 1A). This finding suggested the presence of an acidic Ca^{2+} binding protein (Campbell et al. 1983). This protein migrated in SDS-PAGE with an apparent molecular mass similar to that of CR from different species so far examined (50–60 kDa). Additional bands with lower molecular masses were found to stain blue with Stains All in the same protein fraction.

The Ca^{2+} binding ability of the *Chlamydomonas* protein was confirmed by a [$^{45}Ca^{2+}$] overlay assay. After blotting onto nitrocellulose membrane and incubating with [$^{45}Ca^{2+}$], only the 55-kDa band was labeled (Fig. 1B). The 55-kDa protein showed an acidic isoelectric point (4.9) when analyzed with the O'Farrell (1975) method (Fig. 2).

To obtain further insight into the identity of the 55-kDa protein, we tested its cross-reactivity with specific antibodies to plant and animal CRs. A positive reaction was found with antibodies to spinach (Fig. 3A) and maize (Fig. 3B) CR, whereas no cross-reaction was observed with an antibody to rabbit CR (Fig. 3C). The *Chlamydomonas* Ca^{2+} binding protein was also recognized by an antibody raised against the C-terminal sequence HDEL responsible for the retention of plant CR in the ER. A positive reaction of the 55-kDa band was obtained by blot immunostaining with the specific anti-HDEL monoclonal antibody (Fig. 4). These results led us to identify the 55-kDa band as a CR-like protein because of the presence of three diagnostic properties of high-capacity/low-affinity Ca^{2+} binding proteins (blue staining with Stains All, [$^{45}Ca^{2+}$] binding ability, and an acidic isoelectric point, Milner et al. 1992).

To confirm the identity of the *Chlamydomonas* protein, N-terminal amino acid sequencing of the isolated protein was carried out. The determination of 18 N-terminal amino acids revealed the sequence shown in Figure 5. An identity of about 40% arose from the alignment of the *Chlamydomonas* N-terminal sequence with those of some plant and animal CRs. The degree of identity with *Euglena* CR was found to be higher (50%). This comparison supplied significant evidence that the Ca^{2+}-binding *Chlamydomonas* protein belongs to the CR family.

Glycosylation and in vitro *phosphorylation of Chlamydomonas CR.* It has been reported previously that some plant CRs exhibit high mannose N-linked carbohydrate chains (Navazio et al. 1996). The possibility that *Chlamydomonas* CR could have the same posttranslational modification was investigated by the ligand blot technique with Concanavalin A, a lectin specific for high-mannose oligosaccharides. No detectable labeling of the 55-kDa protein was observed (Fig. 6), indicating that the *Chlamydomonas* Ca^{2+} binding protein is not glycosylated with high-mannose glycans. Furthermore, based on the previously documented *in vitro* phosphorylation by CK2 of spinach CR (Baldan et al. 1996), we investigated whether *Chlamydomonas* CR could be phosphorylated. Negative results were obtained by using protein kinases CK1 and CK2 and a tyrosine kinase (Syk) (Fig. 7) under conditions previously used for spinach CR phosphorylation (Baldan et al. 1996). Simi-

CR	N-terminus																				Reference
Chlamydomonas	K	D	Y	F	K	E	T	F	D	G	S	-	-	W	A	D	R	W	T	K	This paper
Euglena	T	I	Y	Y	K	E	T	F	E	P	D	-	-	W	E	T	R	W	T	H	Navazio et al. 1998
Ricinus	E	V	F	F	E	E	R	F	E	D	G	-	-	W	E	N	R	W	V	K	Coughlan et al. 1997
Tobacco	E	V	F	F	E	E	S	F	N	D	G	-	-	W	E	S	R	W	V	K	Denecke et al. 1995
Barley	D	V	F	F	Q	E	K	F	E	D	G	-	-	W	E	S	R	W	V	K	Chen et al. 1994
Rabbit	V	V	Y	F	K	E	Q	F	L	D	G	D	G	W	T	E	R	W	I	E	Fliegel et al. 1989
Human	A	V	Y	F	K	E	Q	F	L	D	G	D	G	W	T	S	R	W	I	E	McCauliffe et al. 1990

X, undeterminated amino acid residue; -, a gap.
Boxes highlight the similarity among CR sequences.

FIG. 5. N-terminal sequence of *Chlamydomonas* CR compared with the amino acid sequence of different CRs. Alignment of *Chlamydomonas* amino acid N-terminal sequence with homologous CR sequences from different organisms.

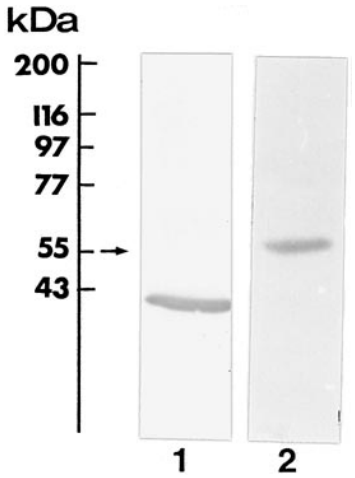

FIG. 6. Affinity detection of high-mannose glycans with concanavalin A. Proteins from the 0.1–0.2 NaCl DEAE-cellulose fraction were separated by SDS-PAGE and blotted onto nitrocellulose membrane. Glycoproteins were detected with the concanavalin A–peroxidase assay. Lane 1, *Chlamydomonas* (15 μg); lane 2, spinach 56-kDa CR (5 μg). Arrow indicates the position of *Chlamydomonas* CR. In lane 1, the 42-kDa band corresponds to an unidentified glycosylated protein present in the DEAE-cellulose fraction.

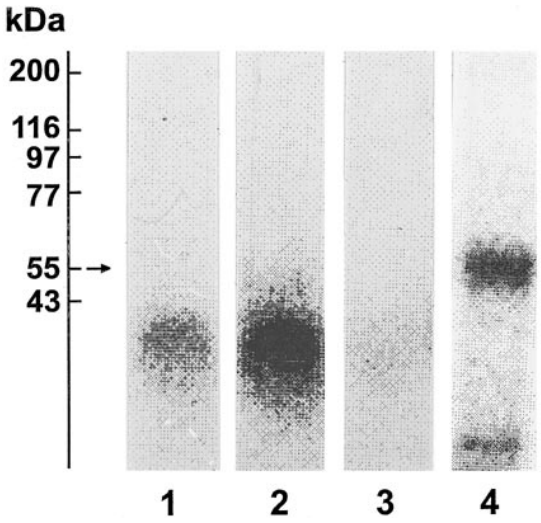

FIG. 7. Phosphorylation of *Chlamydomonas* CR by different protein kinases, detected by Instant Imager. *Chlamydomonas* DEAE-cellulose fraction (17 μg) was incubated in the presence of protein kinases CK1 (lane 1) and CK2 (lane 2) and tyrosine kinase Syk (lane 3). CR from spinach (4 μg) incubated in presence of CK2 was used as positive control (lane 4). Arrow indicates the position of *Chlamydomonas* CR.

lar negative results were obtained when samples were previously treated with AP, and when polylysine (a stimulator of CK2, Meggio et al. 1994) was added to them (data not shown).

CR expression during sexual differentiation of C. reinhardtii. Gametogenesis in *C. reinhardtii* is induced by two major environmental signals: nitrogen starvation and light (Treier et al. 1989, Weissig and Beck 1991). At first the removal in the dark of an utilizable nitrogen source from the culture medium of *Chlamydomonas* vegetative cells induces their differentiation as defined intermediates, the pregametes (Treier and Beck 1991). In a second step, pregametes, which are not able to mate but competent to receive the light signal, can differentiate into mature gametes by a light-induced conversion. Both signals result in a modification of the expression pattern of the cells, with an increase in the rate of synthesis of some proteins (Treier and Beck 1991). In order to analyze the CR expression pattern during the two steps of gametogenesis, total RNA isolated from vegetative cells, pregametes, and gametes was subjected to northern blot analysis using a labeled 800 bp fragment coding for *C. reinhardtii* CR (Zuppini et al. 1999) as a hybridization probe. The results shown in Figure 8A highlighted a CR mRNA accumulation during the two steps of gametogenesis. In particular, CR transcripts increased progressively with progression of gamete differentiation. A quantitative analysis of CR transcripts (Fig. 8B) revealed the same trend in both mating types, with the lowest concentration in vegetative cells. Pregametes and gametes showed an increase in the level of CR mRNA of nearly four and six times, respectively, when compared with CR mRNA level of vegetative cells (Fig. 8B). The high rate of CR synthesis during sexual differentiation was also confirmed by western blot analysis. Crude extracts obtained from vegetative cells, pregametes, and gametes were analyzed on western blot by using an antibody raised against spinach CR. As previously observed for CR mRNA, there was an accumulation of CR during gametogenesis (Fig. 9A). The CR level increased to nearly 25% and 45% in pregametes and gametes, respectively, over the CR level of vegetative cells (Fig. 9B).

We next addressed the question of whether other intraluminal ER proteins could have a differential expression during gamete formation. For this purpose, we investigated the expression of BiP, an ER molecular chaperone involved in the quality control machinery of eukaryotic cells. Western blot analysis on crude extracts obtained from vegetative cells, pregametes, and gametes was carried out using an antibody raised against tobacco BiP that cross-reacted with *C. reinhardtii* BiP (Fig. 10). The semiquantitative analysis pointed to an increase in BiP level in pregametes when compared with vegetative cells, whereas the rate of BiP synthesis remained stable in pregametes and gametes (Fig. 11).

Phylogenetic analysis. The amino acid sequence alignment of *Chlamydomonas* CR cDNA (Zuppini et al. 1999, Genbank accession number AJ000765) with various CR proteins revealed roughly 47% identity with mammals, 52% with *E. gracilis,* and about 55% with higher plants (alignment available upon request). A gene phylogeny of CR proteins (Fig. 12) revealed that *Chlamydomonas* CR assumed a well-supported, basal position among plant CR sequences,

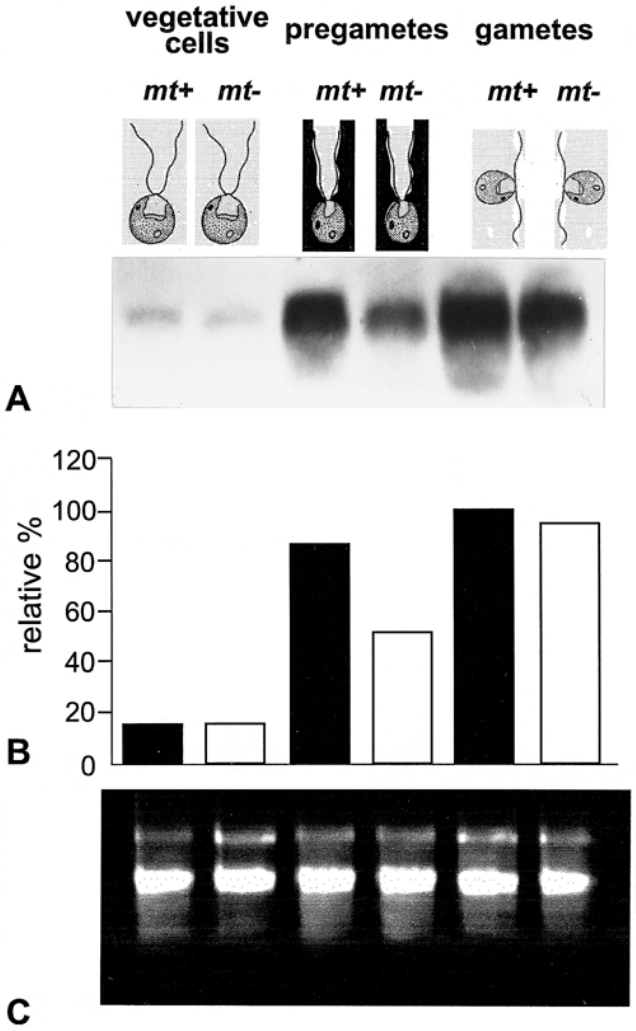

FIG. 8. CR mRNA expression during developmental stages of *C. reinhardtii* gametogenesis. (A) Northern analysis of total RNA (30 μg) extracted from vegetative cells, pregametes, and gametes (autoradiography exposed for 15 min). (B) Densitometry of the exposed film: levels of transcripts were calculated in relative percentage, the higher level of CR mRNA (gametes *mating type*$^+$) was used as 100% reference. (C) Loading control, ethidium bromide staining of the gel.

as would be expected for this unicellular chlorophyte (Melkonian 1996). Database searching with the blast algorithm revealed no homology between CR and any protein sequence from prokaryotic genomes (data not shown).

DISCUSSION

In this paper, we provide evidence that the unicellular chlorophyte *Chlamydomonas reinhardtii* expresses CR, a Ca^{2+} binding protein involved in Ca^{2+} homeostasis in eukaryotic cells. The three diagnostic assays for CR-like proteins supplied us with positive indications of CR in *Chlamydomonas*. A major Stains-All staining 55-kDa band, with an acidic isoelectric point that bound [$^{45}Ca^{2+}$], was found in a DEAE-cellulose purified extract. Immunological investigations revealed a cross-reactivity with antibodies raised against spinach and maize CR, but not with an anti-rabbit CR antibody. The protein isolated from *Chlamydomonas* seems also to reside in the ER, in light of its positive cross-reaction with an antibody produced against the C-terminal retention signal HDEL. This sequence has been found in all plant CRs investigated so far, in contrast to animal CRs, which possess a KDEL ER retention signal at their

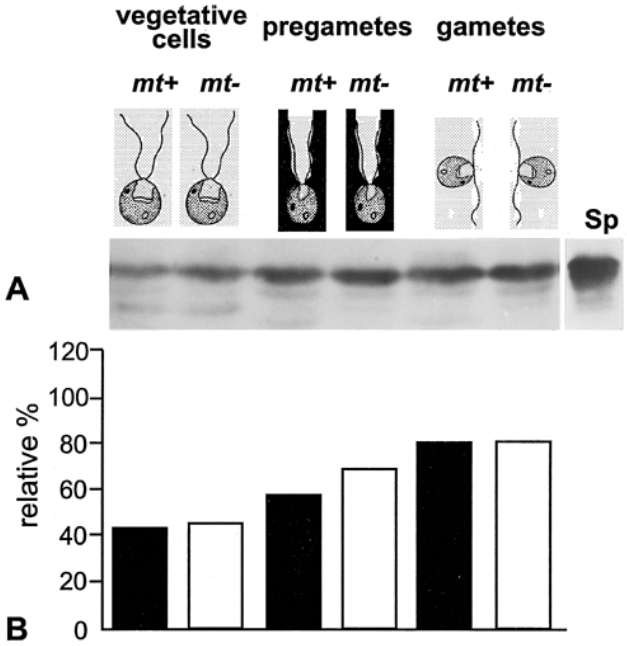

FIG. 9. Western analysis of CR in crude extracts (130 μg) obtained from vegetative cells, pregametes, and gametes. (A) Immunostaining with an antibody to spinach CR (autoradiography exposed for 5 min). CR isolated from spinach leaves (2 μg) was used as reference (lane 7). (B) Densitometry of the exposed film: protein levels were calculated in relative percentage; the higher level of CR (gametes) was used as 100% reference.

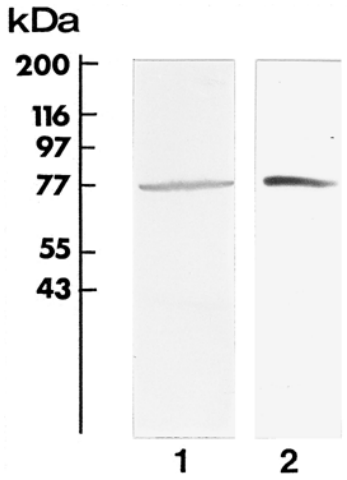

FIG. 10. Immunostaining of *Chlamydomonas* DEAE-cellulose fraction with an antibody to tobacco BiP. Lane 1, *Chlamydomonas* 0.1–0.2 M NaCl DEAE-cellulose fraction (40 μg). Lane 2, 0.2–0.4 M DEAE-cellulose fraction isolated from spinach leaves (13 μg).

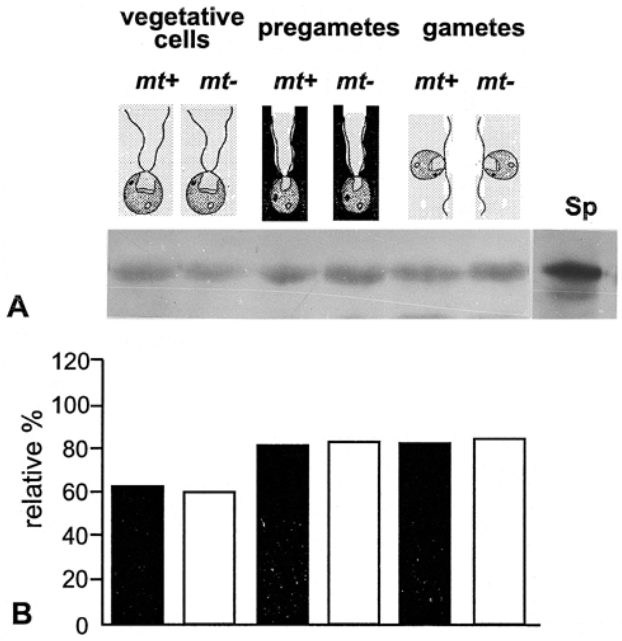

FIG. 11. Western analysis of BiP in crude extracts (130 μg) obtained from vegetative cell, pregametes, and gametes. (A) Immunostaining with an antibody to tobacco BiP (autoradiography exposed for 15 min); 0.2–0.4 M DEAE-cellulose fraction isolated from spinach leaves (2 μg). (B) Densitometry of the exposed film: protein levels were calculated in relative percentage; the higher level of BiP expression (gametes) was used as 100% reference. Sp, 0.2–0.4 M DEAE-cellulose fraction isolated from spinach leaves (13 μg).

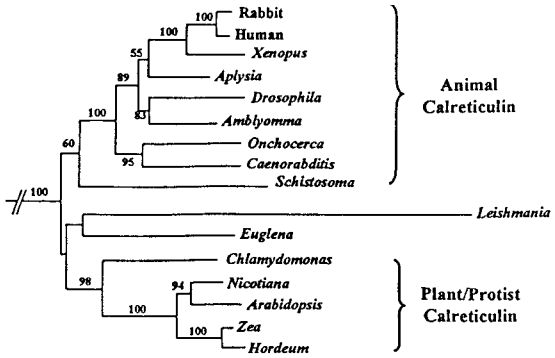

FIG. 12. Phylogeny of CR amino acid sequences. Numbers indicate the bootstrap value for the respective branch (values <50% not indicated). The phylogeny was rooted to the calnexin sequences analyzed in Navazio et al. (1998), as indicated by the leftmost branch.

C-termini (Michalak 1996). That the *Chlamydomonas* protein purified here clearly belongs to the CR family was demonstrated by sequencing the N-terminal region of the protein. In this region, the highest similarity was observed between *Chlamydomonas* and the corresponding region of the *Euglena* protein. The similarity with both animals and other plant homologues was less pronounced.

Most plant CRs seem to share some additional biochemical characteristics, such as glycosylation (Navazio et al. 1996) and *in vitro* phosphorylation (Baldan et al. 1996). *Chlamydomonas* CR was found neither to be N-glycosylated with high-mannose oligosaccharides nor to undergo *in vitro* phosphorylation by protein kinases. In this regard, *Chlamydomonas* CR seems to behave like CR from *Euglena* and animal sources, suggesting that these properties may be specific to CR from higher plants and that these post-translational modifications may have appeared only late in plant evolution. This view is supported by the finding that N-glycosylation has been found only in CRs from angiosperms; CR from the ancient gymnosperm *Ginkgo biloba* is apparently not glycosylated (Nardi et al. 1998).

Recent analyses on plant CR revealed a regulation of CR expression related to sexual reproduction. In barley ovaries, the level of CR transcripts increased immediately after pollination and in the first steps of embryogenesis (Chen et al. 1994). Moreover, a high CR level was measured in maize spermatic cells (Williams et al. 1997), zygotes, and immature embryos (Dresselhaus et al. 1996) and in the floral tissues of *Arabidopsis* (Nelson et al. 1997).

Here we demonstrate that CR expression is developmentally regulated during *Chlamydomonas* gamete formation, with an overall increase of nearly 6 of the CR transcripts and 45% of the protein in mature gametes compared to vegetative cells. The progressive increase of CR level in both pregametes and gametes suggests that CR is involved in the two stages of differentiation. Several studies show that different stresses could induce a higher expression of CR, which in these conditions acts mainly as a molecular chaperone (Denecke et al. 1995, Napier et al. 1995, Heal and McGivan 1998). Withdrawal of a nitrogen source from vegetative cells is undoubtedly a metabolic stress to cells, and the higher rate of CR synthesis could relate to a demand for chaperone-CR. The finding that an increase of the BiP, one of the main ER molecular chaperones, occurs only during the differentiation of pregametes, supports this hypothesis. Then, CR could act mainly as a molecular chaperone during the first stage of differentiation, whereas in the second stage, which prepares cells to mate, CR could have a role as a Ca^{2+} buffer. Ca^{2+} signaling is indeed an integral part of the mating reactions: during gamete formation Ca^{2+} is stored in the intracellular compartments, and it is later released during fertilization (Kaska et al. 1985, Harris 1989, Quarmby 1994). The sequestering of Ca^{2+} ions in rapidly exchanging intracellular stores requires the participation of an efficient Ca^{2+} buffering system, which in the ER relies on Ca^{2+} binding proteins, mainly CR. In this context, an increase of CR expression during gametogenesis might be expected.

The comparison of available CR amino acid sequences from various sources shows regions of both strong conservation and high variability within this protein across eukaryotes. Our phylogenetic analysis reveals two notable findings. First, *Chlamydomonas*

CR does not share a common branch with its homologue from the ER of *Euglena gracilis,* although *Euglena*'s plastids clearly descend from a chlorophytic, eukaryotic symbiont (Martin et al. 1998). This can be attributed to an origin of *Euglena*'s CR from the kinetoplastid host (Navazio et al. 1998), represented here by *Leishmania,* rather than from the unicellular chlorophytic lineage, represented here by *Chlamydomonas.* Second, no significant homology is detected between CR and any prokaryotic sequence. Because eukaryotic genomes are thought to descend from a mixture of eubacterial and archaebacterial genomes (Martin and Müller 1998), this result suggests that some ER-specific proteins such as CR may be evolutionary ''inventions'' that, like the ER-itself, arose only in the eukaryotic lineage and are therefore specific to the eukaryotic lineage.

In light of the ubiquitous distribution of CR among eukaryotic cells and its conservation during evolution, the demonstration that *Chlamydomonas* expresses CR suggests that the intraluminal Ca^{2+} storage machinery of the ER, of which CR is a key component, was already in place in the progenitors of higher plants, but that in certain lineages of higher plants further post-translational modifications were acquired. The degree of conservation within CR homologues underscores the view that once the functional properties of this protein arose in eukaryotic evolution, it was subject to a high level of functional constraint, consistent with its integral role in Ca^{2+} homeostasis and in Ca^{2+}-dependent signalling processes in eukaryotes.

We thank Dr. M. Michalak, Dr. R. M. Napier, and Dr. A. Vitale for kindly providing the antibodies. This work was supported by Ministero Università Ricerca Scientifica e Tecnologica (to P.M.).

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